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The study of microbiologically influenced corrosion (MIC) has progressed from phenomenological case histories to a mature interdisciplinary science including electrochemical, metallurgical, surface analytical, microbiological, biotechnological and biophysical techniques. With gene probes and microelectrodes it is now possible to measure interfacial dissolved oxygen, dissolved sulfide and pH and to further determine the microbial species responsible for the localized chemistry. Biofilms can be tailored to contain consortia of specific microorganisms and naturally occurring biofilms can be dissected into cellular and extracellular constituents. Scanning vibrating electrodes can be used to map the distribution of anodes and cathodes so that localized corrosion can be correlated with the location of microorganisms. The development of environmental scanning electron, atomic force, and laser confocal microscopy makes it possible to image cells on surfaces and to accurately determine the spatial relationship between microorganisms and corrosion. Transport of nutrients through biofilms is being modeled using techniques including optical density measurements to precisely locate the water/biofilm interface and nuclear magnetic resonance imaging to visualize flow characteristics near surfaces colonized with microorganisms. The way in which these new techniques can be used to understand fundamental mechanisms and to discriminate critical issues of MIC will be discussed.

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RECENT ADVANCES IN THE STUDY OF MICROBIOLOGICALLY INFLUENCED CORROSION

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ABSTRACT

The study of microbiologically influenced corrosion (MIC) has progressed from phenomenological case histories to a mature interdisciplinary science including electrochemical, metallurgical, surface analytical, microbiological, biotechnological and biophysical techniques. With gene probes and microelectrodes it is now possible to measure interfacial dissolved oxygen, dissolved sulfide and pH and to further determine the microbial species responsible for the localized chemistry. Biofilms can be tailored to contain consortia of specific microorganisms and naturally occurring biofilms can be dissected into cellular and extracellular constituents. Scanning vibrating electrodes can be used to map the distribution of anodes and cathodes so that localized corrosion can be correlated with the location of microorganisms. The development of environmental scanning electron, atomic force, and laser confocal microscopy makes it possible to image cells on surfaces and to accurately determine the spatial relationship between microorganisms and corrosion. Transport of nutrients through biofilms is being modeled using techniques including optical density measurements to precisely locate the water/biofilm interface and nuclear magnetic resonance imaging to visualize flow characteristics near surfaces colonized with microorganisms. The ways in which these new techniques can be used to understand fundamental mechanisms and to discriminate critical issues of MIC will be discussed.

Introduction

Corrosion associated with microorganisms has been recognized for over 50 years, yet the study of MIC is a relatively new, multidisciplinary field [1, 2]. Several traditionally disparate disciplines have been combined to elucidate the complexities of microbial interactions with metal substrata. Some of the most important developments in MIC research have resulted from nondestructive surface analytical and electrochemical methods to characterize biofilms and electrochemical reactions.

Techniques for Determining Cellular Constituents Within Biofilms

Culture techniques

For many years, the standard for evaluating MIC has been the enumeration of sulfate-reducing bacteria (SRB), either in bulk liquids or in surface deposits, using a liquid medium with sodium lactate as the carbon source [3, 4]. When SRB are present in the sample, sulfate is reduced to sulfide, which reacts with iron in solution to produce black ferrous sulfide. Blackening of the medium over a 28-day period signals the presence of SRB. Usually, 1 ml samples are injected by syringe into media bottles for 10-fold

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dilutions. It is assumed that only a single living bacterium is required to blacken a bottle. The simplest interpretation of test results is to consider that if one bottle is blackened, the sample contained at least one organism; if two bottles are blackened, the sample contained 10 organisms; three bottles, 100 organisms and so on. Solid media can also be used [5]. Agar slants can be inoculated by dipping a pipe cleaner into a liquid sample and inserting it into a single vial of solid or semisolid agar. Mineral oil and a CO_2-generating tablet are usually added to exclude oxygen and the vial is capped, incubated for 5 days, and checked daily for blackening.

The distinct advantage of culturing techniques is that they are extremely sensitive. Low numbers of SRB grow to easily detectable higher numbers in the proper culture medium. However, growth media tend to be strain-specific. For example, lactate-based media sustain the growth of lactate oxidizers but not acetate-oxidizing bacteria. Incubating at one temperature is further selective. Culturing methods using agar media cannot distinguish between a single SRB cell and a clump of SRB cells [5]. The present trend in culture techniques is to attempt to culture several physiological groups including aerobic, heterophic bacteria; facultative anaerobic bacteria; and acid-producing bacteria in addition to sulfate-reducing bacteria [6]. A complex SRB medium has been developed, containing multiple carbon sources that can be degraded to both acetate and lactate. In recent comparison tests, the complex medium produced higher counts of SRB in naturally produced waters and in surface deposits among five commercially available media [7].

Biochemical Assays

Biochemical assays have been developed for the detection of specific microorganisms associated with MIC. Unlike culturing techniques, biochemical assays for detecting and quantifying bacteria do not require growth of the bacteria. Instead, biochemical assays measure constitutive properties including adenosine triphosphate (ATP) [8], phospholipid fatty acids (PLFA) [2], cell-bound antibodies [9, 10], and DNA [11]. Adenosine-5'-phosphosulfate (APS) reductase [5], hydrogenase [12], and radiorespirometric measurements have been used to estimate SRB populations and activity [13, 14].

ATP assays estimate the total number of viable organisms by measuring the amount of adenosine triphosphate in a sample. ATP is a compound found in all living matter. The procedure requires that a water sample be filtered to remove solids and salts that may interfere with the test. The filtered sample is added to a reagent that releases cell ATP. An enzyme then reacts with the ATP to produce a photochemical reaction. Emitted light can be measured with a photometer and the number of bacterial cells is estimated from the total light emitted.

Biofilm community structure can be analyzed through cluster analysis of the phospholipid fatty acid profiles [2]. PLFA profiles for natural biofilms have been shown to be more complex than profiles for laboratory biofilms. None of the laboratory profiles clustered closely with profiles from natural biofilms. In addition, the PLFA profiles for attached bacteria clustered separately from profiles of the same bacteria in the bulk phase, suggesting that either the community or the physiology of attached bacteria differ from that of bulk phase bacteria.

Immunofluorescence techniques have been developed for the identification of specific bacteria in biofilms [15, 16]. Epifluorescence cell surface antibody (ECSA) methods for detecting SRB are particularly well-developed and are based on the use and subsequent detection of specific antibodies, produced in rabbits, that react with SRB cells [9, 10]. A secondary antibody, produced in goats, is then reacted with the primary rabbit antibodies bound to the SRB cells. In some cases, goat antibodies are linked to a fluorochrome

that enables bacterial cells marked with the secondary antibody to be viewed with an epifluorescence microscope. In other cases, goat antibodies are conjugated with an enzyme (alkaline phosphatase) that can then be reacted with a colorless substrate to produce a visible color proportional to the quantity of SRB present. The detection limits for the field test are 10,000 SRB per ml. The color reagent used for the field tests is unstable at room temperature and the color reagent tends to bind nonspecifically with antibodies adsorbed directly at active sites on the filter, creating a false positive that may interfere with the detection of SRB at levels below 10,000 cells per ml. Antigenic structures of marine and terrestrial strains are distinctly different and therefore antibodies to either strain did not react with the other. Furthermore, SRB antibodies did not react with non-SRB bacteria. The developers report a poor response of rabbit antibodies developed from pure SRB cultures to mixed populations [10]. Rabbit SRB antibodies generated from fresh SRB strains from Prudhoe Bay, Alaska, as well as terrestrial and marine locations, were found to react better with SRB from natural sources. It is possible to differentiate individual species within a biofilm by reacting them with monoclonal antibodies specific to outer cell membrane antigens. Hogan has described a nonisotopic semiquantitative procedure for the detection of *Desulfobacterium* and *Desulfotomaculum*, using DNA probes labeled with an acridinium ester that is sensitive to 10^4 organisms per ml [11].

Direct molecular characterization of natural microbial populations can be accomplished with sequence analysis of 5S rRNAs [17, 18]. More recently, fluorescent dye-labeled oligonucleotide probes have been used for microscopic identification of single cells and characterization of mixed populations. Polymerase chain reaction amplification, comparative sequencing and whole cell hybridization have been combined to selectively identify and visualize sulfate-reducing bacteria both in established and developing multispecies biofilms [19].

APS reductase is an intercellular enzyme found in all SRB. Briefly, cells are washed to remove interfering chemicals including hydrogen sulfide and lysed to release APS reductase. The lysed sample is washed, added to an antibody reagent and exposed to a color-developing solution. In the presence of APS reductase, a blue color appears within 10 minutes. The degree of color is proportional to the amount of enzyme and roughly to the number of cells from which the enzyme was extracted. Similarly, a procedure has been developed to quantify hydrogenase from SRB that requires that cells be concentrated by filtration from water samples [12]. Solids, including corrosion product and sludge, can be used without pretreatment. The sample is exposed to an enzyme for 15 minutes and placed in an anaerobic chamber from which oxygen is removed by hydrogen. The enzyme reacts with excess hydrogen and simultaneously reduces an indicator dye in solution. The activity of the hydrogenase is established by the development of a blue color in less than 4 hours. The intensity of the blue color is proportional to the rate of hydrogen uptake by the enzyme. The technique does not attempt to estimate specific numbers of SRB.

Roszak and Colwell [20] reviewed the techniques commonly used to detect microbial activities in natural environments, including transformations of radiolabelled metabolic precursors. Phelps et al. [21] and Mittelman et al. [22] used uptake or transformation of ^{14}C-labelled metabolic precursors to examine the activities of sessile bacteria in natural environments and in laboratory models. Phelps et al. [21] used a variety of ^{14}C-labelled compounds to quantify catabolic and anabolic bacterial activities associated with corrosion tubercles in natural gas transmission pipelines. They demonstrated that organic acid was produced from H_2 and CO_2 in natural gas by acetogenic bacteria, and that acidification could lead to enhanced corrosion of the steel. Mittelman et al. [22] used measurement of lipid biosynthesis from ^{14}C-acetate, in conjunction with measurements

of microbial biomass and extracellular polymer, to study the effects of differential fluid shear on the physiology and metabolism of *Alteromonas* (formerly *Pseudomonas*) *atlantica*. Increasing shear force increased the rate of total lipid biosynthesis, but decreased cell biosynthesis. Increasing fluid shear also increased cellular biomass and greatly increased the ratio of extracellular polymer to cellular protein.

Techniques for analyzing microbial metabolic activity at localized sites are also being developed. Franklin et al. [23] incubated microbial biofilms with ^{14}C-metabolic precursors and then autoradiographed the biofilms to localize biosynthetic activity on corroding metal surfaces. The localized uptake of the labelled compounds was related to localized electrochemical activities associated with corrosion reactions.

A major breakthrough in determining bacterial activity within biofilms has been the use of "reporter" genes that can signal when the activity of specific metabolic pathways are induced. King et al. [24] engineered the incorporation of a promotorless cassette of *lux* genes into specific operons of *Pseudomonas* so that these operons induce bioluminescence during the degradation of naphthalene. Mittelman et al. [25] have used the bioluminescent reporter gene to provide a quantitative measure of the attachment of these organisms onto metal and glass surfaces in a laminar flow system. They found that biofilm light production was directly correlated with biofilm cell numbers in a range of 10^5–10^7 cells cm^{-2}. Using reporter genes, Marshall et al. [26] demonstrated that bacteria immobilized at surfaces exhibit physiological properties that are not found in the same organisms in the aqueous phase. Some genes are turned on at a solid surface despite not being expressed in liquid or on solid media. It is also likely that other genes are turned off at surfaces. They have identified acid- and alkali-inducible genes in *E. coli*. Marshall et al. [26] further demonstrated gene transfers within biofilms even in the absence of any imposed selection pressure.

Rosser and Hamilton [13], with subsequent modifications [14], developed a test tube technique to perform a ^{35}S sulfate radiorespirometric assay to measure SRB metabolic activity on the surface of metal coupons after exposure to corrosive environments. The coupon is placed into anaerobic filtered sterile seawater containing ^{35}S-sulfate. Oxygen-free zinc acetate is immediately injected onto an enclosed filter paper wick and the entire system is incubated. Oxygen-free hydrochloric acid is then injected past the wick into the solution. Volatile acid sulfides, including any $H_2{}^{35}S$ formed, are trapped during an equilibration period. The wick is removed from the tube and the radioactivity is measured by using a liquid scintillation counter, after which the sulfate reduction rate is calculated. This technique has been used for both bulk and coupon samples.

Bacteria within biofilms are protected from antimicrobial agents, including biocides. Bacteria are generally resistant to 50 to 500 times the biocide concentration sufficient to kill planktonic or free floating cells of the same species. Blenkinsopp et al. [27] discovered that the application of a low strength electric field current density can enhance the efficacy of glutaraldehyde to such an extent that bacteria within a biofilm are killed at biocide concentrations lower than the planktonic minimum inhibitory concentration. This enhanced biocide efficacy in the presence of an electric field/current density has been called the "bioelectric effect."

Techniques for Identification and Measurement of MIC

Electrochemical Techniques

Mansfeld and Little [28] recently reviewed electrochemical techniques applied to MIC studies and no attempt will be made to discuss all the innovations in electrochemical

techniques. However, two nondestructive electrochemical techniques, the scanning vibrating electrode technique (SVET) and electrochemical impedance spectroscopy (EIS) are being used in several laboratories to provide unique insights into mechanisms for MIC.

SVET has been used to monitor development of corrosion and changes in corrosion rate [29]. More recently the SVET has been applied to MIC. The SVET uses a vibrating electrode to map potential fields in solution over anodic and cathodic sites. Corrosion currents depend on polarization characteristics of metal surfaces. The vibrating electrode converts potential fields into an alternating signal. Franklin et al. [30] used the SVET to localize corrosion on carbon steel associated with MIC and to demonstrate a spatial relationship between localized corrosion and bacterial cells. They demonstrated that in sterile, continuously stirred media, small pits initiated on steel surfaces and subsequently repassivated. Initiated pits remained active and propagated in the presence of biofilms. Pit propagation depended on the presence of bacteria. The authors were careful not to conclude that bacterial cells caused pits. Instead, bacterial cells prevented repassivation and were clearly spatially related to the pits. The authors did propose that biofilms may inhibit migration of aggressive ions from the pits or migration of inhibiting ions from the bulk solution into pits. The sensitivity and the resolution of the SVET are determined by the distance between probe and metal surface in addition to solution conductivity. A decrease in the tip diameter increases the sensitivity and resolution of the technique. However, a very fine tip has a very high impedance that increases the electrochemical noise and decreases the response time. Resolution also depends on the proximity of two corroding sites and the magnitude of the corrosion current from each site.

EIS has been used to study the impedance of metal samples exposed to microbial biofilms [31]. With EIS small amplitude sinusoidal signals over a frequency range (10 kHz to 5 mHz) are applied to metal samples and the amplitude and the phase shift of the resulting sinusoidal currents are measured. Interfacial impedance can be modeled by using electrical circuits containing resistors and capacitors in series and in parallel. The spectra of responses provide information associated with corrosion. EIS can provide values of polarization resistance (which is inversely proportional to the corrosion rate), double layer capacitance, and solution resistance. Using EIS data, models have been prepared for localized corrosion and three-dimensional surface deposits. EIS studies have been used to determine the impact of changes in community structure on corrosion of mild steel. Jack used a flow-through freshwater aerobic system with a combination of strict aerobes, facultative anaerobes, and strict anaerobes to show that specific combinations of bacteria were more important to corrosion than total numbers of any specific cell type [32].

Surface Analytical Techniques

Nivens et al. [33] demonstrated that attenuated total reflectance infrared spectroscopy (ATR-FT/IR) can be used to detect changes in sessile microbial biomass. The ATR-FT/IR studies showed that changes in the physiological properties of the attached bacteria were induced by changes in the bulk phase. They demonstrated that the number of attached *Caulobacter sp.* was directly correlated with the intensity of the infrared amide II asymmetrical stretch band at 1543 cm^{-1}, corresponding to bacterial protein. The technique was sensitive to 10^6 bacteria per cm^{-2}, and changes in the physiological status of the attached bacteria could be measured. For example, production of the intracellular storage lipid, poly-B hydroxyalkanoate, and production of extracellular polymer, were monitored by absorbance at 1730 cm^{-1} (C=O stretch) and 1084 cm^{-1} (C–O stretch), respectively.

Geesey et al. [34] used ATR-FT/IR not only to detect biofilm formation, but also evaluated nondestructively in real-time interactions of bacteria isolated on thin films of copper evaporated onto germanium internal reflection elements. By sputtercoating a thin film of copper on the germanium internal reflectance element, they were able to detect changes in the thickness of the copper films by observing the increase in intensity of the infrared water absorption band at 1640 cm^{-1}. The authors compared copper loss from copper thin films in the presence of bacteria isolated from corroded copper samples. Different rates of metal loss were observed in two cultures. Using this technique, Jolley et al [35] observed copper loss in the absence of bacteria caused by the binding by bacterial extracellular polymer. Differences on the order of 0.3 to 0.4 nm, the equivalent of two to three atomic layers of copper can be detected.

Nivens et al. [33] investigated the use of the quartz crystal microbalance (QCM), a very sensitive mass-sensing device, for detecting attached microbial films. The QCM was more sensitive to changes in biomass than the ATR-FT/IR, with a detection limit of 10^4 bacteria cm^{-2} and a linear range of at least two orders of magnitude. An interesting aspect of both the ATR-FT/IR and the QCM is that substrata of both techniques can be converted to electrodes for electrochemical analyses so that corrosion information can be obtained while changes in microbial biofilms are monitored.

It is now generally recognized that biofilms alter biofilm/metal interfacial chemistries. Direct chemical measurements are restricted by biofilm thickness and the heterogeneous and anisotropic nature of biofilms [36]. Ion-selective and gas-sensing microelectrodes with tip diameters less than 10 μm are being used for these direct measurements. Lewandowski [36] has measured dissolved oxygen profiles in a continuous flow open-channel reactor with a mixed biofilm on a metal surface. Van Houdt et al. [37] developed a sputtered iridium oxide pH microelectrode with a tip diameter in the range of 3 to 15 μm. This microelectrode was used to measure a pH profile across a mixed population biofilm on a polycarbonate disc.

An in-situ microtechnique has been developed for evaluating parameters of diffusion-controlled reactions in biofilms [38]. A microprobe 15 μm in diameter was used to simultaneously measure dissolved oxygen and optical density at different depths in a submerged biofilm. The diffusion coefficient for dissolved oxygen, the dissolved oxygen flux, and the half velocity coefficient were then calculated.

Nuclear magnetic resonance imaging (NMRI) is a noninvasive method using radiofrequency magnetic fields in the presence of a strong static magnetic field to learn from an assembly of specific atomic nuclei about concentration and physical state such as flow and chemical environment. Lewandowski et al. [39] demonstrated the use of NMRI to show distribution of water, flow velocities, and biomass in a biofilm/polycarbonate reactor system.

Recent developments in image analysis systems and electron, atomic, and laser microscopy make it possible to image biological materials in the hydrated state. Muellar et al. [40] were able to determine rate coefficients for early bacterial colonization on copper, silicon, stainless steel and glass using a chemostat, a flow cell and a microscope equipped with an image analysis system. Reflective substrata were monitored using reflective light from a microscope equipped with a Nomarski lens and microscope output was linked to a video camera. Transmitted light was used for transparent surfaces. They demonstrated that surface roughness and surface free energy correlated positively with biological and abiological sorption processes.

Little et al. [41] used environmental scanning electron microscopy (ESEM) to study biofilms, comparing results to those seen using traditional scanning electron microscopy (SEM). ESEM images of wet estuarine biofilms on stainless steel surfaces indicated

a gelatinous layer in which numerous algae were located. Many microorganisms produce extracellular polymeric acidic polysaccharides that can bind and precipitate heavy metals. The estuarine biofilm on stainless steel surfaces accumulated local concentrations of Al, Ni, and Ti. Dehydration of the biofilm with solvents is required in sample preparation for SEM. This either extracts bound metals from the biofilm by ion exchange/solvent extraction or removes the metals with the extracellular polymeric material. The solvent effects cannot be separated from the mechanical disturbance to the biofilms during sample manipulation.

Laser confocal microscopy permits one to create three-dimensional images, see surface contour in minute detail and accurately measure critical dimensions by mechanically scanning the object with laser light [42]. A sharply focused image of a single horizontal plane within a specimen is formed while light from out of focus areas is repressed from view. The process is repeated again and again at precise intervals on horizontal planes and the visual data from all images are compiled to create a single, multidimensional view of the subject. Geesey [43] used laser confocal microscopy to produce three-dimensional images of bacteria within scratches, milling lines and grain boundaries.

The atomic force microscope (AFM) was derived from the scanning tunneling microscope (STM). The STM uses an atomically sharp conductive tip held angstroms from the surface in order to profile surface features with angstrom resolution. When the tip is electrically biased with respect to the sample a current will flow between the surface atom closest to the tip and the nearest tip atom through the quantum mechanical process of electron tunneling. Current is exponentially sensitive to the distance between the tip and the sample. A piezoelectric transducer is used to mechanically raster scan the tip over the sample. AFM provides exceptional detail and allows viewing of specimens in the hydrated state. AFM uses a scanning probe to record x,y,z coordinates of a sample in fractions of a nanometer. Photodiode electrical outputs mimic sample topography and serve as the basis for the resulting image. AFM images of copper exposed to bacterial culture medium for seven days showed biofilms distributed heterogeneously across the surface with regard to both cell numbers and depth [44]. Bacterial cells were associated with pits on the surface of the copper coupons.

Conversion of metals to sulfides by SRB has been studied since the late 1800s [45]. Baas-Becking and Moore have identified mackinawite, gregrite and smythite as indicators for SRB corrosion of ferrous metals in anaerobic environments [46]. McNeil et al. analyzed sulfide mineral deposits on copper alloys colonized by SRB in an attempt to identify specific mineralogies that could be used to fingerprint SRB activity [47]. They concluded that the formation of nonadherent layers of chalcocite (Cu_2S) and the presence of hexagonal chalcocite were indicators of SRB-induced corrosion of copper. The compounds were not observed abiotically and their presence in near-surface environments could not be explained thermodynamically.

Sulfur isotope fractionation was demonstrated by Little et al. in sulfide corrosion deposits resulting from the activities of SRB within biofilms on copper surfaces [48]. ^{32}S accumulated in sulfide-rich corrosion products. ^{34}S was concentrated in the residual sulfate in the culture medium. Conventionally, the amplitude of each isotope is not reported individually, but a ratio is established and compared with the isotope ratio of a standard to yield a value $\delta^{34}S$, expressed as parts per thousand. Negative $\delta^{34}S$ values indicate a concentration of ^{32}S, and positive values indicate an accumulation of $\delta^{34}S$. Accumulation of the lighter isotope was related to surface derivatization or corrosion as measured by weight loss. Use of this and the preceding mineralogical technique to identify SRB-related corrosion requires sophisticated laboratory procedures.

Conclusion

The study of MIC has matured into a recognized multidisciplinary research area in which the combined approaches of electrochemists, materials scientists, surface chemists and microbiologists are providing insight into complex interactions between biofilms and materials.

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